

IND-6, a Highly Divergent IND-Type Metallo- β -Lactamase from *Chryseobacterium indologenes* Strain 597 Isolated in Burkina Faso[∇]

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The genus *Chryseobacterium* and other genera belonging to the family *Flavobacteriaceae* include organisms that can behave as human pathogens and are known to cause different kinds of infections. Several species of *Flavobacteriaceae*, including *Chryseobacterium indologenes*, are naturally resistant to β -lactam antibiotics (including carbapenems), due to the production of a resident metallo- β -lactamase. Although *C. indologenes* presently constitutes a limited clinical threat, the incidence of infections caused by this organism is increasing in some settings, where isolates that exhibit multidrug resistance phenotypes (including resistance to aminoglycosides and quinolones) have been detected. Here, we report the identification and characterization of a new IND-type variant from a *C. indologenes* isolate from Burkina Faso that is resistant to β -lactams and aminoglycosides. The levels of sequence identity of the new variant to other IND-type metallo- β -lactamases range between 72 and 90% (for IND-4 and IND-5, respectively). The purified enzyme exhibited N-terminal heterogeneity and a posttranslational modification consisting of the presence of a pyroglutamate residue at the N terminus. IND-6 shows a broad substrate profile, with overall higher turnover rates than IND-5 and higher activities than IND-2 and IND-5 against ceftazidime and cefepime.

Metallo- β -lactamases (MBLs) belong to Ambler's class B, and their activity requires at least one zinc ion in the active site. These enzymes are particularly worrisome resistance determinants due to their ability to hydrolyze most β -lactam compounds, including carbapenems, while they are not susceptible to conventional β -lactamase inhibitors (e.g., clavulanic acid, sulbactam, and tazobactam) (25). Besides the acquired MBL genes which currently disseminate in major human pathogens (e.g., *Pseudomonas aeruginosa*) by means of mobile genetic elements (plasmids, integrons, or ISCR elements), most MBL genes identified thus far have been found in the chromosomes of various bacterial species, including nonpathogenic organisms (such as *Caulobacter crescentus*), plant pathogens (*Erwinia carotovora*), and occasional human pathogens such as *Stenotrophomonas maltophilia*, *Aeromonas* spp., and members of the family *Flavobacteriaceae* (25, 32). The latter family includes several species in which the production of a resident MBL has been detected. Although their overall clinical impact is lower than that of *Enterobacteriaceae*, *Pseudomonas*, or *Acinetobacter* species, *Flavobacteriaceae* may cause various clinical syndromes that are not always straightforward to treat, due mainly to their intrinsic resistance to several antimicrobial agents (29). Eight different sublineages of resident enzymes in members of the family *Flavobacteriaceae* have been identified thus far (4–7, 18, 20, 26), and most of them (BlaB from *Elizabethkingia meningoseptica*, IND from *Chryseobacterium indologenes*, JOHN

from *Flavobacterium johnsoniae*, CGB from *Chryseobacterium gleum*, EBR from *Empedobacter brevis*, TUS from *Myroides odoratus*, and MUS from *Myroides odoratimimus*) belong to subclass B1, although the GOB enzymes (from *E. meningoseptica*) belong to subclass B3.

The most common flavobacterium from clinical specimens is *C. indologenes*, which is associated with different types of infections, such as intra-abdominal and urinary tract infections, catheter-related bacteremia, cellulitis, sepsis, and pneumonia, likely promoted by the bacterium's ability to form biofilm and to produce proteases (1, 3, 15, 16, 21, 23, 24, 33). In some nosocomial settings, *C. indologenes* infections were also associated with relatively high mortality rates (16). Six variants of resident MBLs (IND-1 to IND-5 and IND-2a) have been detected in *C. indologenes*, and these variants diverge by at most 27% (IND-2a versus IND-4) at the protein sequence level (6, 8, 22). The biochemical characterization of these enzymes revealed interesting functional differences regarding affinities for various substrates, turnover rates (with up to 30-fold variation between the rates of two variants for the same substrate), and their abilities to hydrolyze ceftazidime or cefepime (8, 22). In this work, we report the isolation of a *C. indologenes* strain from Burkina Faso that caused a urinary tract infection and produces a new IND-type variant, named IND-6, for which detailed biochemical characterization was performed.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *C. indologenes* strain 597 was obtained from a urine sample from an outpatient at the Saint Camille Medical Center, Ouagadougou, Burkina Faso (34), and was used as the source of the MBL gene. *Escherichia coli* DH5 α (Gibco Life Technologies, Gaithersburg, MD) was used as a host for recombinant plasmids, while *E. coli* BL21(DE3) (Strat-

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agene, La Jolla, CA) was used for overproduction of the IND-6 enzyme by a T7 promoter-based expression system. Bacteria were always grown aerobically. *C. indologenes* and *E. coli* DH5 α derivative strains were cultured at 37°C in Mueller-Hinton broth (Oxoid Ltd., Basingstoke, United Kingdom). ZYP-0.8G medium was used for routine propagation of *E. coli* BL21(DE3) derivatives, while ZYP-5052 medium was used for production of the recombinant protein (30).

Molecular and recombinant DNA methodologies. The complete sequence of the MBL gene from *C. indologenes* 597 was obtained using an inverse PCR approach, as described previously by Bellais and coworkers (8). Genomic DNA was obtained using the standard alkaline-sodium dodecyl sulfate lysis method (27), and a 4- μ g sample was digested with restriction endonucleases BamHI, ClaI, PstI, and SalI. After complete digestion, restriction fragments were circularized by the addition of 1 U of T4 DNA ligase in the buffer system supplied by the manufacturer (Roche Biochemicals, Mannheim, Germany). PCR was then carried out as described previously (8) using circularized restriction fragments from genomic DNA as the template, primers IND-INV/+ (5'-TTGGCAGAAT ATTCTTTACC) and IND-INV/- (5'-GAAAAAAGACGGAAAGCAAC), and 3 U of *Tth* DNA polymerase (Promega, Carlsbad, CA). The resulting amplified fragments were cloned into plasmid pMOSBlue (GE Healthcare, Uppsala, Sweden), and both strands were sequenced using T7 promoter and M13 to M20 universal primers (27).

The *bla*_{IND-6} open reading frame (ORF) was subsequently amplified by PCR with a primer (5'-GGGCATATGAAAAGAAGAAATTCAGTTC) that added an NdeI restriction site (underlined) to the 5' end and primer IND-EXP/r (5'-CC GGATCCTTATTATTCTTATCCAGCAGC), which added a BamHI restriction site (underlined) to the 3' end of the gene. PCR was performed with 5 U of the Expand high-fidelity PCR system DNA polymerase in accordance with the instructions of the manufacturer (Roche Biochemicals) by using 200 μ M deoxynucleoside triphosphates, 50 pmol of each primer, and 100 ng of *C. indologenes* 597 genomic DNA as the template in a total volume of 50 μ l. Cycling conditions were as follows: an initial denaturation step at 96°C for 3 min, 30 cycles of denaturation at 96°C for 40s, annealing at 53°C for 40 s, and extension at 72°C for 2 min, and a final extension step at 72°C for 20 min. The amplified DNA was cloned into vector pLB-II (a derivative of pBC-SK [Stratagene, La Jolla, CA] modified in our laboratory) (9), yielding recombinant plasmid pLBII-IND-ZB. After confirmatory sequencing, the 720-bp NdeI-BamHI fragment was subcloned into the expression vector pET-9a (Novagen, Madison, WI) to obtain recombinant plasmid pET9-IND-ZB.

Antimicrobial susceptibility testing. The in vitro antimicrobial susceptibility profiles of *C. indologenes* 597 and *E. coli* DH5 α derivatives were determined by the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) (10) by using Mueller-Hinton broth with a bacterial inoculum of 5×10^4 CFU/well. MICs were recorded after 18 h at 37°C.

Production and purification of IND-6. The cloned MBL of *C. indologenes* was purified from a culture of *E. coli* BL21(DE3)(pET-IND-ZB) grown for 24 h at 37°C in 1 liter of ZYP-5052 medium supplemented with 50 μ g/ml kanamycin. The culture supernatant, containing most of the β -lactamase activity, was clarified by centrifugation (10,000 \times g for 30 min at 4°C), concentrated using an Amicon 2000 ultrafiltration device equipped with a YM10 membrane (Millipore, Bedford, MA), and desalted using a HiPrep 26/10 desalting column (GE Healthcare, Uppsala, Sweden) and a solution of 10 mM HEPES-NaOH containing 50 μ M ZnSO₄ (pH 7.5; HZN buffer) as the elution buffer. The resulting sample was loaded at a flow rate of 2 ml/min onto an SP Sepharose high-performance column (bed volume, 5 ml; GE Healthcare), and bound proteins were eluted using a linear NaCl gradient (0 to 1 M in 100 ml). The β -lactamase-containing fractions were diluted 10-fold in 10 mM MES (morpholineethanesulfonic acid) buffer (pH 6.0) supplemented with 50 μ M ZnSO₄ and loaded onto a Resource S column (bed volume, 1 ml) preequilibrated with the same supplemented buffer. Bound proteins were eluted using a linear NaCl gradient in the same buffer (0 to 0.5 M in 25 ml), and β -lactamase-containing fractions were pooled, concentrated to 0.25 mg/ml, and stored at -20°C until further use.

Protein analysis techniques. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, isoelectric focusing, and determination of protein concentrations in solution were performed as described previously (11). The molecular mass of the native IND-6 enzyme was estimated by size exclusion chromatography using a Superdex 75 HR 10/30 column (GE Healthcare) with HZN buffer supplemented with 150 mM NaCl as described previously (11). Electrospray ionization mass spectrometry analysis of the purified enzyme (final concentration, 15 μ M) was carried out using a quadrupole time of flight (TOF) Ultima mass spectrometer (Micromass, Manchester, United Kingdom) equipped with a nanospray source, as described previously (12). Peptide mass fingerprint analysis was performed after digestion of the protein sample with either trypsin or ArgC endoproteinase by using an Ultraflex II TOF/TOF mass spectrometer (Bruker

Daltonics, Bremen, Germany). Resulting data were analyzed with Biotoools 3.0 software (Bruker Daltonics). The putative structure of IND-6 was computed using the homology modeling service available at the ExPASy/SwissProt website (Swiss-Model Workspace [http://swissmodel.expasy.org/workspace/]) with the crystal structure of BlaB1 (Protein Data Bank code 1M2X) as the starting model (2, 14).

Determination of kinetics parameters and inactivation by chelating agents. The hydrolysis of β -lactam substrates was monitored by measuring the absorbance variation under the experimental conditions reported previously (11). All measurements were performed with a Cary 100 UV-vis spectrophotometer (Varian, Walnut Creek, CA) or an Uvikon XL spectrophotometer (Kontron Instruments, Eching, Germany) at 30°C using HZN buffer in a reaction volume of 500 μ l. Purified IND-6 was diluted in HZN buffer supplemented with 20 μ g/ml bovine serum albumin to prevent enzyme denaturation. The steady-state kinetic parameters (k_{cat} and K_m) were calculated after direct fitting of the initial rates to the Henri-Michaelis-Menten equation or with the use of Hanes-Woolf linearization. The inactivation of IND-6 by EDTA and pyridine-2,6-dicarboxylic (dipicolinic) acid at 30°C in 50 mM HEPES buffer (pH 7.5) was studied using 150 μ M imipenem as the reporter substrate. Kinetic parameters of inactivation were computed as described previously (11).

Nucleotide sequence accession number. The nucleotide sequence determined in this study has been submitted to the EMBL/GenBank/DBBL sequence databases and assigned the accession number AM087455.

RESULTS AND DISCUSSION

Susceptibility profile of and β -lactamase production in *C. indologenes* 597. The *C. indologenes* 597 isolate was recovered from a urine sample from a 25-year-old female outpatient at the Saint Camille Hospital Centre in Ouagadougou, Burkina Faso. No evidence of previous antibiotic treatments was recorded. Determination of the antimicrobial susceptibility profile showed that *C. indologenes* 597 was resistant to several β -lactam agents, including ampicillin, amoxicillin (amoxicilline)-clavulanic acid, cephalothin (cefalotin), cefuroxime, and imipenem (MICs, ≥ 64 μ g/ml), and notably to all tested aminoglycosides (amikacin MIC, ≥ 64 μ g/ml; gentamicin and tobramycin MICs, ≥ 16 μ g/ml). The strain also showed decreased susceptibilities to cefoxitin, cefotaxime, and meropenem (MICs, 16, 32, and 8 μ g/ml, respectively), while it was susceptible to piperacillin, piperacillin-tazobactam, ceftazidime, cefepime, all tested quinolones (ciprofloxacin, norfloxacin, and ofloxacin [MICs, ≤ 2 μ g/ml]), and a folate metabolism inhibitor (trimethoprim-sulfamethoxazole) (Table 1). Hydrolysis tests performed with various β -lactam substrates (including ampicillin, cephalothin, cefotaxime, and imipenem) and a crude extract of *C. indologenes* 597 demonstrated the production of a β -lactamase, the activity of which could be inhibited >95% after incubation with 5 mM EDTA, indicating the production of an MBL most likely of the IND type. Nitrocefin hydrolysis after isoelectric focusing revealed the presence of a single β -lactamase band at pI 9.0, suggesting that other β -lactamases, including serine-active enzymes, were not produced at a significant level by this strain.

Molecular cloning of *bla*_{IND-6}, a new IND-type MBL variant. Due to sequence heterogeneity at the 5' and 3' extremities of the MBL gene, direct amplification with consensus primers designed to amplify all known IND-like MBL genes (*bla*_{IND-1} to *bla*_{IND-5}) was unsuccessful. On that basis, the inverse PCR approach was adopted to obtain the complete sequence of the *bla*_{IND}-like ORF, as done previously by Bellais and coworkers (8). By using circularized restriction fragments of genomic DNA as the template in the PCR, an amplification fragment (approximately 1 kb) was obtained from the samples digested

TABLE 1. In vitro susceptibility profiles of *C. indologenes* 597, *E. coli* DH5 α (pLBII-IND-ZB) carrying the cloned *bla*_{IND-6} gene, and *E. coli* DH5 α carrying the empty plasmid

β -Lactam	MIC (μ g/ml) for:		
	<i>C. indologenes</i> 597	<i>E. coli</i> DH5 α (pLBII-IND-ZB)	<i>E. coli</i> DH5 α (pBC-SK)
Ampicillin	≥ 64	32	2
Amoxicillin-clavulanic acid	≥ 64	ND ^a	ND
Piperacillin	4	ND	ND
Piperacillin-tazobactam	4	ND	ND
Ticarcillin	ND	256	4
Temocillin	ND	16	4
Cephalothin	≥ 64	64	4
Cefoxitin	16	16	2
Cefuroxime	≥ 64	32	2
Cefotaxime	32	0.5	0.12
Ceftazidime	8	1	0.12
Cefepime	1	0.12	0.03
Ceftriaxone	ND	0.12	0.03
Imipenem	≥ 64	1	0.12
Meropenem	8	0.12	0.03
Aztreonam	ND	0.25	0.25

^a ND, not determined.

with the ClaI restriction endonucleases and was cloned into plasmid pMOSBlue to yield recombinant vector pIND-597. Sequencing of the cloned PCR fragment allowed the determination of the nucleotide sequence of the whole *bla*_{IND-6}-like ORF (723 bp) and approximately 300 bp of the upstream flanking region.

The product of the *bla*_{IND} ORF showed the highest sequence similarities to IND-1, IND-3, and IND-5 variants (identity scores computed using the complete protein sequence ranged from 88.3 to 89.5%) but also showed 11 unique substitutions, thus constituting a new IND variant, named IND-6 (Fig. 1 and 2), that also increases the maximum sequence divergence among IND-type variants (to 28.5%, between IND-4 and IND-6). Of these 11 unique substitutions, 10 were

located in the mature protein and most were concentrated in the N-terminal domain between Asp-120 and His-196 (Pro-140, Gln-144, Arg-148, Pro-176, and Phe-182) or in the C-terminal domain between Cys-221 and His-263 (Leu-239, His-247, Glu-255, and Val-257) (Fig. 1 and 3).

No other putative ORFs could be found immediately upstream of the *bla*_{IND-6} ORF, as reported previously (6, 8), although comparison of the available 300-bp nucleotide sequence with the sequence found upstream of the *bla*_{IND-1} gene showed significant heterogeneity (identity, 71%), and comparison with the sequence upstream of the *bla*_{CGB} gene also showed some extent of heterogeneity. Another difference concerns the nature of the putative promoter found upstream of the MBL gene, which possesses a mutation in the -35 signal (ttGcta, where the uppercase letter indicates the position of the mutation) compared to the -35 promoter sequence found upstream of the *bla*_{IND-1} gene (ttCcta) and may explain, in addition to individual catalytic features of the various IND-type enzymes, the different levels of intrinsic resistance to β -lactams exhibited by various *C. indologenes* strains, as reported recently (17).

After subcloning of the *bla*_{IND-6} ORF into a proper vector system (i.e., one carrying a resistance marker other than ampicillin resistance), which was introduced into *E. coli*, the antimicrobial susceptibility profile of the host was determined, showing that the production of IND-6 resulted in increased MICs of all tested antibiotics except aztreonam, which is not a substrate for MBLs (Table 1). The production of IND-6 in *E. coli* did not confer resistance (according to CLSI breakpoints) to carbapenem antibiotics (10). These data are in agreement with those reported for *E. coli* laboratory strains producing other MBL determinants (4–8, 18, 20, 22, 26) and should be related to the fast permeation of these agents into the host, as demonstrated previously (19).

Purification and biophysical characterization of IND-6. The MBL was successfully produced by the strategy adopted for the production of other MBLs (11, 12), i.e., using a T7 promoter-

TABLE 2. Kinetic properties of the purified IND-6 MBL with various β -lactam compounds^a

Substrate	k_{cat} (s^{-1}) for IND-6	K_m (μ M) for IND-6	k_{cat}/K_m value ($M^{-1} s^{-1}$) for:			
			IND-6	IND-2	IND-5	BlaB1
Benzylpenicillin	305	20	1.5×10^7	4.6×10^6	8.4×10^5	2.7×10^7
Amoxicillin	480	85	5.6×10^6	— ^b	—	—
Carbenicillin	210	30	7.0×10^6	—	5.3×10^4	6.1×10^6
Cephalothin	415	60	6.9×10^6	1.2×10^6	—	1.6×10^6
Cephaloridine	490	90	5.4×10^6	—	—	1.4×10^5
Cephalexin	19	3	6.3×10^6	—	—	—
Cefuroxime	75	23	3.3×10^6	—	—	1.0×10^6
Cefotaxime	8	33	2.4×10^5	9.0×10^5	3.1×10^4	7.9×10^5
Ceftazidime	6	22	2.7×10^5	4.5×10^3	ND ^{d,e}	6.6×10^3
Cefepime	>1.2	>300	4.0×10^3	4.5×10^2	ND ^{d,e}	2.0×10^2
Nitrocefin	115	43	2.7×10^6	—	—	3.9×10^6
Imipenem	50	13	3.8×10^6	5.9×10^5	2.4×10^4	2.0×10^6
Meropenem	25	145	1.7×10^5	1.8×10^5	1.5×10^4	7.6×10^5
Aztreonam	NH ^c	ND ^d	ND ^d	ND ^d	ND ^d	ND ^d

^a Standard deviations were below 10%. k_{cat}/K_m values for IND-2, IND-5, and BlaB1 are shown for comparison (8, 22, 26).

^b —, data not available.

^c NH, no hydrolysis detected with enzyme concentrations up to 0.9 μ M.

^d ND, not determined.

^e Ceftazidime and cefepime are not hydrolyzed by IND-5 (22).

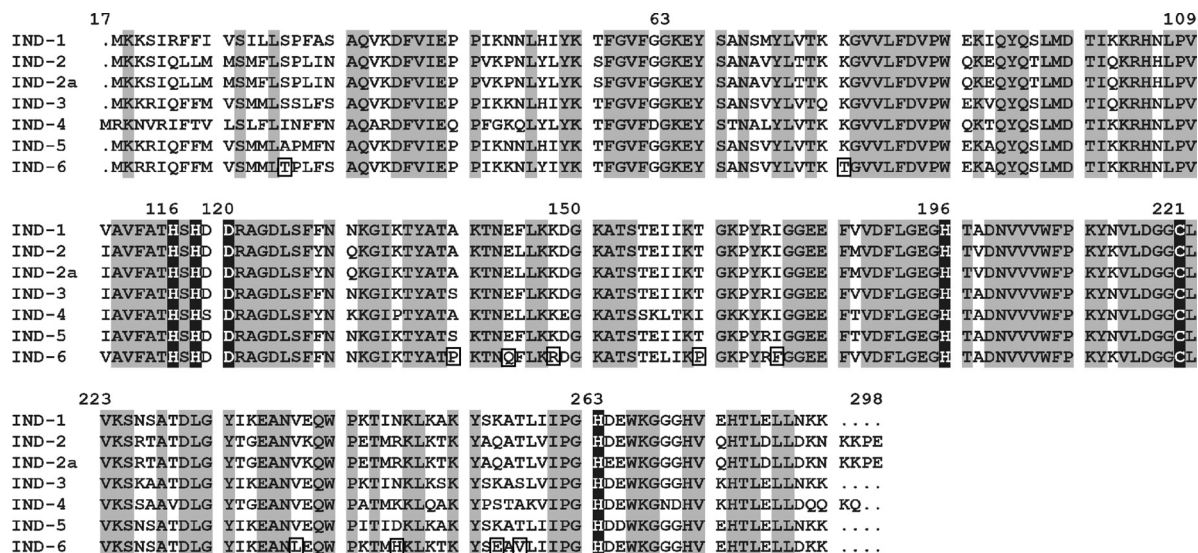


FIG. 1. Alignment of multiple amino acid sequences from IND variants (IND-1 [accession no. AAD20273], IND-2 [accession no. AAG29757], IND-2a [accession no. AAG29760], IND-3 [accession no. AAG29762], IND-4 [accession no. AAG29765], and IND-5 [accession no. AAS78754]). The new IND variant from *C. indologenes* 597 exhibits the closest relationship with IND-5 (89.5% pairwise sequence identity), while being more divergent from IND-4 (71.5% identity). The standard numbering scheme for class B β -lactamases has been used (13). Identical residues are shaded in gray, and the conserved zinc-binding residues of subclass B1 MBLs are shaded in black. Unique substitutions found in IND-6 are boxed.

based *E. coli* expression system, which yielded approximately 2 mg of recombinant protein per liter of culture. The purification scheme involved two chromatographic steps and yielded an enzyme preparation with a purity of >95%, with a global yield of approximately 25% (this apparently low yield from the purification protocol is explained by the fact that only the purest fractions were kept for subsequent analysis). The apparent molecular mass of the enzyme in solution ($M_r = 25,000 \pm 5,000$) determined by size exclusion chromatography indicated that the enzyme in solution is monomeric. Electrospray ionization mass spectrometry analysis of the purified sample revealed the presence of four distinct enzyme species (corresponding to masses of 24,707.4, 24,725.9, 24,794.0, and

24,882.6 Da; standard deviation, 3 Da), suggesting the presence of either amino- or carboxy-terminal heterogeneity or other kinds of posttranslational modifications. Peptide mass fingerprint and matrix-assisted laser desorption/ionization-TOF mass spectrometry analyses revealed that peptides showing mass heterogeneity could be assigned to the N-terminal extremity of the protein. The higher-mass species, which represented only a minor isoform, corresponded to the mature protein obtained by cleavage of an 18-residue signal peptide (theoretical mass, 24,884.5 Da), while two lower-mass species (24,794.0 and 24,725.9 Da) corresponded to alternative cleavage sites after positions 19 and 20, respectively. These three forms thus correspond to the deduced N-terminal sequences S¹⁹AQVK, A²⁰QVKD, and Q²¹VKDF. The lowest-mass species, presenting an additional loss of 18 mass units, was attributed to the cyclization of the N-terminal glutamine to form a pyroglutamate residue. This posttranslational modification is common in proteins and may have biological implications, as proteins bearing an N-terminal pyroglutamate residue exhibit greater stability against proteolysis (28).

Biochemical features of IND-6. The purified enzyme hydrolyzed all the tested β -lactam substrates (except aztreonam), showing a broad substrate profile (Table 2). The highest k_{cat}/K_m values were observed with penicillins, narrow-spectrum cephalosporins, cefuroxime, and imipenem (k_{cat}/K_m values, $>10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$). Cefotaxime, ceftazidime, and meropenem were roughly 50- to 90-fold less reactive than benzylpenicillin. Cefepime behaved as the worst substrate, although the observed k_{cat}/K_m values were higher than those measured with other IND variants (i.e., IND-2 and IND-5, for which kinetic parameters have been measured) or with BlaB1 (8, 22, 26). The latter was also true for ceftazidime, which showed a 60-fold-higher k_{cat}/K_m value with IND-6 than with IND-2 (4,500

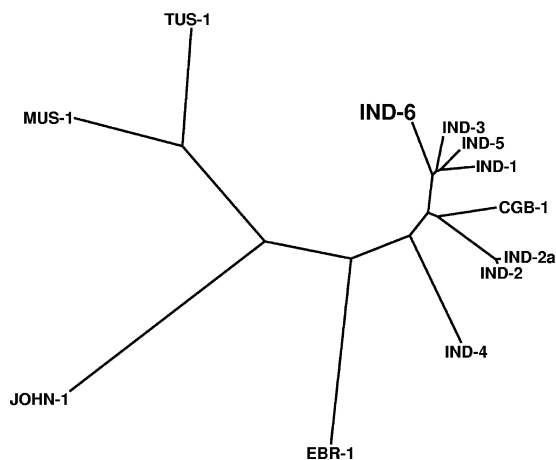


FIG. 2. Unrooted tree showing the phylogenetic relationships of IND-6 with other MBLs identified in bacterial species belonging to the family *Flavobacteriaceae* (accession numbers of sequences are as given in the legend to Fig. 1).

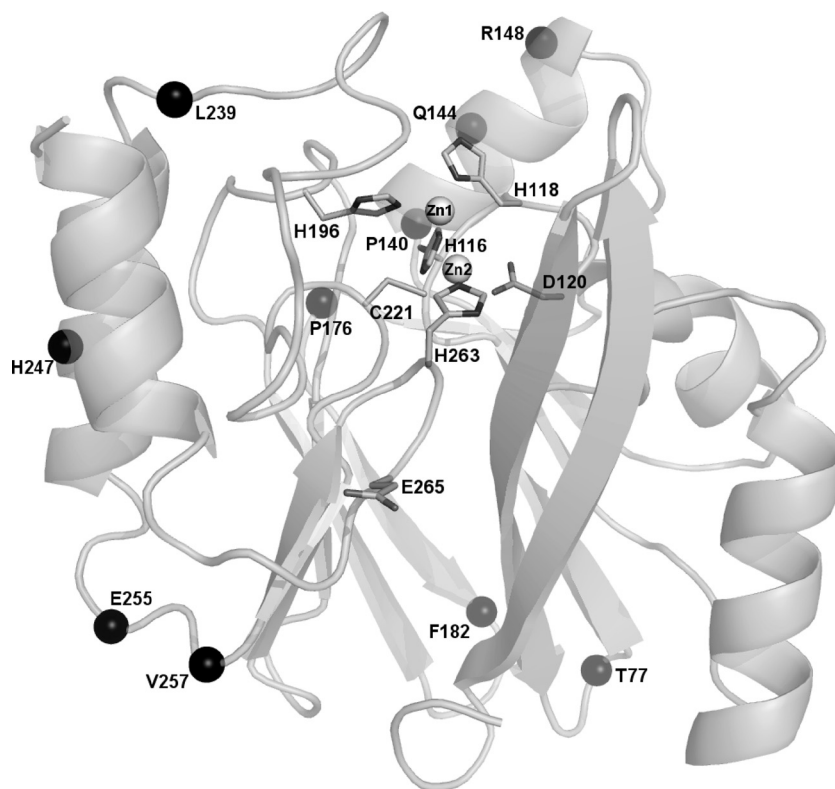


FIG. 3. Cartoon representation of the IND-6 homology model showing the putative tertiary structure and the locations of amino acid substitutions discussed in the text. The positions of unique substitutions found in IND-6 are shown as black spheres; the position of residue E265, located close to the active-site residue H263 and replaced with aspartate in IND-5 and BlaB1, is also shown.

$M^{-1} \cdot s^{-1}$), while this substrate was not recognized by IND-5 (8, 22).

The highest turnover rates were observed with penicillins and narrow- and expanded-spectrum cephalosporins (k_{cat} values, $\geq 75 s^{-1}$), except for cephalexin (cefalexin), which interestingly showed a much lower k_{cat} value than cephalothin or cephaloridine (Table 2). This finding likely reflects the presence of an amino group on the C-7 side chain and the absence of a leaving group on the C-3 carbon, which would be responsible for slower hydrolysis of this compound (Fig. 4). IND-6 also exhibited lower K_m values for ceftazidime, imipenem, and meropenem than other IND variants, indicating better recognition of these substrates. Overall, IND-6 exhibited functional properties that are very different from those of IND-5, despite the fact that these two enzymes share 90% amino acid sequence identity, as summarized by the higher reactivity of IND-6 toward ceftazidime and cefepime (which are not hydrolyzed by IND-5) and overall much higher turnover rates. These results may further support the hypothesis that the substitution E265D occurring in IND-5 may be detrimental for the hydrolysis of some substrates (22). Position 265 is located only one residue away from the zinc-binding His-263 residue, and it may be hypothesized that a substitution in the loop where the latter residue is found may influence the position of its side chain, possibly inducing subtle changes in the geometry of the zinc-2 site and thus the activity of the enzyme. The potential effect of remote mutations on enzyme activity was already described for another model enzyme (31). Interestingly, the same substitu-

tion (E265D) is also found in the BlaB1 enzyme, which also shows lower catalytic efficiencies with ceftazidime and cefepime. Nevertheless, it is likely that substitutions other than E265D are responsible for the overall properties of IND-6, as reflected by the fact that IND-2, which does not exhibit the E265D substitution, also shows differences from IND-6 in terms of catalytic efficiencies with some substrates.

In inactivation experiments, IND-6 showed greater suscep-

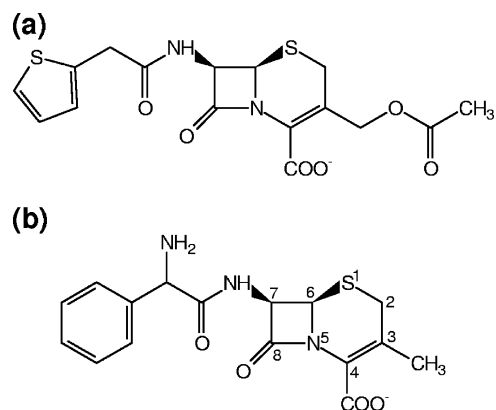


FIG. 4. Comparison of the structures of cephalothin (cefalotin) (a) and cephalexin (cefalexin) (b), showing the nonreplaced methyl group at the 3 position and the presence of an amino group on the side chain at the 7 position in the latter.

tibility to dipicolinic acid than to EDTA. As observed for other subclass B1 MBLs (11, 25), the inactivation rates varied proportionally with the inactivator concentration and only the pseudo-first-order constant, which reflects the inactivation efficiency, could be determined (k_{+2}/K values, 9.4 and 170 $M^{-1} \cdot s^{-1}$ for EDTA and dipicolinic acid, respectively, where k_{+2} is the individual rate constant for the dissociation of the ternary Zn-enzyme-chelator complex into the apoenzyme and the metal-chelator complex and K is the dissociation constant of the ternary complex in the model proposed by Hernandez-Valladeres et al. [15a]). These values were similar to those observed with other subclass B1 enzymes (11, 25).

Concluding remarks. *C. indologenes* is increasingly reported to be a fastidious organism, involved in many different types of infections. Its intrinsic resistance to most β -lactam agents, including carbapenems, and occasional association with resistance to aminoglycosides and fluoroquinolones may limit the available options for successful antimicrobial treatment (18). In this work, we described the new resident MBL determinant IND-6, identified in an isolate from Burkina Faso, which showed particularly high hydrolytic activities against β -lactam substrates. The apparent contrast between the catalytic properties of the enzyme and the antimicrobial susceptibility profile of the original host (which showed susceptibility to ceftazidime and cefepime and only intermediate resistance to meropenem) most likely results from a low level of expression of the MBL determinant. This variability in the resistance pattern has also recently been observed between strains producing identical MBL determinants (namely, IND-1 or IND-2), supporting the idea that alterations in the promoter sequence may influence the resistance level (18). In addition, differences in the permeability of the outer membrane or the sensitivity of the penicillin-binding proteins may be factors in resistance (although these issues have not been specifically investigated in the present study), as hypothesized previously and in agreement with the fact that *C. indologenes* isolates commonly exhibit resistance to aztreonam, which is not a substrate of MBLs (18). Should this species be more and more frequently encountered in the clinical setting, the questions of (i) whether β -lactams could be successfully used against susceptible strains and (ii) whether the apparently variable susceptibility profile would rely on the acquisition of another resistance mechanism(s) would deserve attention. Finally, the sequence heterogeneity shown by IND-type enzymes, which leads to natural variants showing different biochemical properties, makes these enzymes interesting models to investigate the role of remote mutations in MBL structure and function.

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REFERENCES

- Akay, M., E. Gunduz, and Z. Gulbas. 2006. Catheter-related bacteremia due to *Chryseobacterium indologenes* in a bone marrow transplant recipient. *Bone Marrow Transplant.* **37**:435–436.
- Arnold, K., L. Bordoli, J. Kopp, and T. Schwede. 2006. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* **22**:195–201.
- Bayraktar, M. R., E. Aktas, Y. Ersoy, A. Cicek, and R. Durmaz. 2007. Postoperative *Chryseobacterium indologenes* bloodstream infection caused by contamination of distillate water. *Infect. Control Hosp. Epidemiol.* **28**:368–369.
- Bellais, S., D. Aubert, T. Naas, and P. Nordmann. 2000. Molecular and biochemical heterogeneity of class B carbapenem-hydrolyzing β -lactamases in *Chryseobacterium meningosepticum*. *Antimicrob. Agents Chemother.* **44**:1878–1886.
- Bellais, S., D. Girlich, A. Karim, and P. Nordmann. 2002. EBR-1, a novel Ambler subclass B1 β -lactamase from *Empedobacter brevis*. *Antimicrob. Agents Chemother.* **46**:3223–3227.
- Bellais, S., S. Leotard, L. Poirel, T. Naas, and P. Nordmann. 1999. Molecular characterization of a carbapenem-hydrolyzing β -lactamase from *Chryseobacterium (Flavobacterium) indologenes*. *FEMS Microbiol. Lett.* **171**:127–132.
- Bellais, S., T. Naas, and P. Nordmann. 2002. Genetic and biochemical characterization of CGB-1, an Ambler class B carbapenem-hydrolyzing β -lactamase from *Chryseobacterium gleum*. *Antimicrob. Agents Chemother.* **46**:2791–2796.
- Bellais, S., L. Poirel, S. Leotard, T. Naas, and P. Nordmann. 2000. Genetic diversity of carbapenem-hydrolyzing metallo- β -lactamases from *Chryseobacterium (Flavobacterium) indologenes*. *Antimicrob. Agents Chemother.* **44**:3028–3034.
- Borgianni, L., J. M. Frere, G. M. Rossolini, and J. D. Docquier. 2006. Mutational analysis of the VIM-2 active site: role of positions 64 and 87 in enzyme activity and stability, abstr. C1-28, p. 63–64. Abstr. 46th Intersci. Conf. Antimicrob. Agents Chemother., San Francisco, CA.
- Clinical and Laboratory Standards Institute. 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard, 7th ed. CLSI document M7–A7. Clinical and Laboratory Standards Institute, Wayne, PA.
- Docquier, J. D., J. Lamotte-Brasseur, M. Galleni, G. Amicosante, J. M. Frere, and G. M. Rossolini. 2003. On functional and structural heterogeneity of VIM-type metallo- β -lactamases. *J. Antimicrob. Chemother.* **51**:257–266.
- Docquier, J. D., F. Pantanella, F. Giuliani, M. C. Thaller, G. Amicosante, M. Galleni, J. M. Frere, K. Bush, and G. M. Rossolini. 2002. CAU-1, a subclass B3 metallo- β -lactamase of low substrate affinity encoded by an ortholog present in the *Caulobacter crescentus* chromosome. *Antimicrob. Agents Chemother.* **46**:1823–1830.
- Galleni, M., J. Lamotte-Brasseur, G. M. Rossolini, J. Spencer, O. Dideberg, and J. M. Frere. 2001. Standard numbering scheme for class B β -lactamases. *Antimicrob. Agents Chemother.* **45**:660–663.
- Garcia-Saez, I., J. Hopkins, C. Papamicael, N. Franceschini, G. Amicosante, G. M. Rossolini, M. Galleni, J. M. Frere, and O. Dideberg. 2003. The 1.5-Å structure of *Chryseobacterium meningosepticum* zinc β -lactamase in complex with the inhibitor, D-captopril. *J. Biol. Chem.* **278**:23868–23873.
- Green, B. T., and P. E. Nolan. 2001. Cellulitis and bacteraemia due to *Chryseobacterium indologenes*. *J. Infect.* **42**:219–220.
- Hernandez-Valladeres, M., A. Felici, G. Weber, H. W. Adolph, M. Zeppezaer, G. M. Rossolini, G. Amicosante, J. M. Frere, and M. Galleni. 1997. Zn(II) dependence of the *Aeromonas hydrophila* AE036 metallo- β -lactamase activity and stability. *Biochemistry* **36**:11534–11541.
- Hsueh, P. R., L. J. Teng, P. C. Yang, S. W. Ho, W. C. Hsieh, and K. T. Luh. 1997. Increasing incidence of nosocomial *Chryseobacterium indologenes* infections in Taiwan. *Eur. J. Clin. Microbiol. Infect. Dis.* **16**:568–574.
- Lin, X. H., Y. H. Xu, J. Cheng, T. Li, and Z. X. Wang. 2008. Heterogeneity of bla_{IND} metallo- β -lactamase-producing *Chryseobacterium indologenes* isolates detected in Hefei, China. *Int. J. Antimicrob. Agents* **32**:398–400.
- Mammeri, H., S. Bellais, and P. Nordmann. 2002. Chromosome-encoded β -lactamases TUS-1 and MUS-1 from *Myroides odoratus* and *Myroides odoratimimus* (formerly *Flavobacterium odoratum*), new members of the lineage of molecular subclass B1 metalloenzymes. *Antimicrob. Agents Chemother.* **46**:3561–3567.
- Matsumura, N., S. Minami, Y. Watanabe, S. Iyobe, and S. Mitsuhashi. 1999. Role of permeability in the activities of β -lactams against gram-negative bacteria which produce a group 3 β -lactamase. *Antimicrob. Agents Chemother.* **43**:2084–2086.
- Naas, T., S. Bellais, and P. Nordmann. 2003. Molecular and biochemical characterization of a carbapenem-hydrolyzing β -lactamase from *Flavobacterium johnsoniae*. *J. Antimicrob. Chemother.* **51**:267–273.
- Pan, H. J., L. J. Teng, Y. C. Chen, P. R. Hsueh, P. C. Yang, S. W. Ho, and K. T. Luh. 2000. High protease activity of *Chryseobacterium indologenes* isolates associated with invasive infection. *J. Microbiol. Immunol. Infect.* **33**:223–226.
- Perilli, M., B. Caporale, G. Celenza, C. Pellegrini, J. D. Docquier, M. Mez-

- zatesta, G. M. Rossolini, S. Stefani, and G. Amicosante. 2007. Identification and characterization of a new metallo- β -lactamase, IND-5, from a clinical isolate of *Chryseobacterium indologenes*. *Antimicrob. Agents Chemother.* **51**:2988–2990.
23. Ray, P., K. Sharma, and V. Gautam. 2005. *Chryseobacterium indologenes* bacteremia: a case report. *J. Commun. Dis.* **37**:259–260.
 24. Reynaud, I., V. Chanteperdrix, C. Broux, P. Pavese, J. Croize, M. Maurin, J. P. Stahl, and C. Jacquot. 2007. A severe form of *Chryseobacterium indologenes* pneumonia in an immunocompetent patient. *Med. Mal. Infect.* **37**:762–764.
 25. Rossolini, G. M., and J. D. Docquier. 2007. Class B β -lactamases, p. 115–144. In R. A. Bonomo and M. E. Tolmasy (ed.), *Enzyme-mediated resistance to antibiotics: mechanisms, dissemination, and prospects for inhibition*. ASM Press, Washington, DC.
 26. Rossolini, G. M., N. Franceschini, M. L. Riccio, P. S. Mercuri, M. Perilli, M. Galleni, J. M. Frere, and G. Amicosante. 1998. Characterization and sequence of the *Chryseobacterium (Flavobacterium) meningosepticum* carbapenemase: a new molecular class B β -lactamase showing a broad substrate profile. *Biochem. J.* **332**(Pt. 1):145–152.
 27. Sambrook, J., and D. W. Russell. 2001. *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
 28. Schilling, S., C. Wasternack, and H. U. Demuth. 2008. Glutaminyl cyclases from animals and plants: a case of functionally convergent protein evolution. *Biol. Chem.* **389**:983–991.
 29. Schreckenberger, P. C., M. I. Daneshvar, and D. G. Hollis. 2007. *Acinetobacter*, *Achromobacter*, *Chryseobacterium*, *Moraxella*, and other nonfermentative gram-negative rods, p. 770–802. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of clinical microbiology*, 9th ed. ASM Press, Washington, DC.
 30. Studier, F. W. 2005. Protein production by auto-induction in high density shaking cultures. *Protein Expr. Purif.* **41**:207–234.
 31. Tomatis, P. E., R. M. Rasia, L. Segovia, and A. J. Vila. 2005. Mimicking natural evolution in metallo- β -lactamases through second-shell ligand mutations. *Proc. Natl. Acad. Sci. USA* **102**:13761–13766.
 32. Walsh, T. R., M. A. Toleman, L. Poirel, and P. Nordmann. 2005. Metallo- β -lactamases: the quiet before the storm? *Clin. Microbiol. Rev.* **18**:306–325.
 33. Wang, S. L., W. T. Hsu, T. W. Liang, Y. H. Yen, and C. L. Wang. 2008. Purification and characterization of three novel keratinolytic metalloproteases produced by *Chryseobacterium indologenes* TKU014 in a shrimp shell powder medium. *Bioresour. Technol.* **99**:5679–5686.
 34. Zeba, B., P. J. Simpoire, O. G. Nacoulma, and J. M. Frere. 2005. Identification of metallo- β -lactamase from a clinical isolate at Saint Camille Hospital Center of Ouagadougou/Burkina Faso. *Afr. J. Biotechnol.* **4**:286–288.